CELL AND TISSUE CULTURE

BY

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PREFACE

THIS book was written in an attempt to provide an up-to-date account of the techniques and applications of tissue culture since, due to the remarkably rapid development of the subject in the last few years, the excellent texts prepared by other authors are already outdated. It is based on the instruction given at the Tissue Culture Association Summer Course and has been written particularly with the needs of participants of that course in mind. The post-doctorate group taking the course represent workers in a wide variety of fields in which the technique is used. Because of their varied backgrounds, there is often a great lack of uniformity in their basic biological knowledge. Thus, mitosis is often a mystery to a biochemist while the Krebs cycle is only a name to an embyrologist. For this reason I have included a few chapters which give an elementary outline of fundamental cytology.

I am of the opinion that a textbook should try to create a sense of proportion and that it fails if it treats all subjects with equal emphasis. Consequently, while giving a brief outline of the entire field of tissue culture, I have tried to emphasise standard procedures. For the reader who might require more detailed information I have provided a reasonably selective bibliography, quoting only key references. The Bibliography of the Research in Tissue Culture is available for those who might wish a complete list of publications.

Undoubtedly, many people will disagree with my opinions as to which subjects require emphasis and it is not unlikely that errors in the text will have escaped my notice. I should like to receive any comments, suggestions and criticisms that readers of the book might wish to make.

It has often been said that a man never writes a book alone and this was never more true than in the present case. I particularly owe a debt to Drs. Margaret Murray and Charles Pomerat who taught me most of what I know about the subject and much of whose material is incorporated in the text. It also includes a great deal of material which has been contributed to the Tissue Culture Course instruction by my colleagues and friends, past and

present. Drs. Payne, Sidman, Ackermann, Rutter, Algard, Black and McCarty, Miss Marilyn Bozeman and Mrs. Elsa Zitcer will all find small sections of this book very familiar but I know none of them will mind the liberty I have taken. I should like not only to acknowledge their contributions but also to thank them for the enthusiastic co-operation that has made the Tissue Culture Course so enjoyable and so worthwhile. There is, in addition, some material in this book which originated from our predecessors on the staff of the Tissue Culture Course and I should like to acknowledge the contributions made by Drs. Hanks, Leighton, Fawcett, Scherer and Porter. For some information incorporated in the virology section I have to thank Dr. R. G. Sommerville.

In the actual compilation of the book I could not have done without the conscientious and enthusiastic help of Mrs. Patricia Kent and I am deeply grateful to her for her secretarial assistance. To Mr. R. Callander I am also extremely grateful for the meticulous care and outstanding draughtsmanship with which he executed the drawings. I should like too to thank Mr. Charles Macmillan and Mr. James Parker of Messrs. E. & S. Livingstone for their help, patience and co-operation.

Professor J. N. Davidson and Miss E. S. Pearson read the manuscript and made numerous useful suggestions which I greatly appreciate.

I gratefully acknowledge the loan of illustrations and blocks by the following: Adelphi Manufacturing Co. Ltd., London; Baird & Tatlock (London) Ltd., London; Becton Dickinson & Company, Rutherford, New Jersey, U.S.A.; Bellco Glass Inc., Vineland, New Jersey, U.S.A.; The Biochemical Journal, London, and Professor H. McIlwain, London; Electro-Mechanical Development Co., Houston, Texas, U.S.A.; Gallenkamp & Co., Ltd., London; Hanovia Lamps, Slough; Kontes Glass Company, Vineland, New Jersey, U.S.A.; Laboratory Thermal Equipment Ltd., Greenfield; Merck Sharp & Dohme Ltd., Hoddesdon, and Dr. Charles Pomerat; Shandon Scientific Co. Ltd., London.

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Glasgow, 1959.

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INTRODUCTION

CHAPTER I

DEVELOPMENT OF TISSUE CULTURE TECHNIQUES

TISSUE culture developed quite naturally from some of the techniques of embryology that were in use last century. Wilhelm Roux's experiment of maintaining the medullary plate of a chick embryo in warm saline for a few days was performed in the year 1885 and is the first recorded instance of a successful explantation. About the same time Arnold (1887) implanted fragments of alder pith into frogs. When these had become invaded by leucocytes he removed them to a dish of warm saline and subsequently observed that they migrated and survived for a short time.

These two experiments were performed ahead of their time and the possibility that excised animal tissues might be kept alive even longer in favourable conditions was not explored until 1898 when Ljunggren demonstrated by reimplantation that human skin could survive *in vitro* for many days if stored in ascitic fluid.

In 1903, Jolly performed experiments which marked the first detailed observations on cell survival and cell division *in vitro*. He maintained leucocytes from the salamander in hanging drops for up to a month. This study was followed three years later, in 1906, by a paper by Beebe and Ewing which recorded a genuine attempt at tissue culture. These authors described the cultivation of an infectious canine lymphosarcoma in blood from resistant and susceptible animals.

Many of these earlier experiments anticipated by thirty or forty years techniques which are in general use today. At the time they were difficult to repeat since the media used were generally unsatisfactory and there was some doubt whether they demonstrated genuine survival of healthy tissues or merely somewhat delayed death of the cells.

It was because Ross Harrison's experiment in 1907 demonstrated quite unequivocal continuation of normal function *in vitro* and

offered a reproducible technique that it has been generally accepted as marking the true beginning of tissue culture. Harrison explanted small pieces of tissue from the medullary tube region of frog embryos into clots of frog lymph. When kept in aseptic conditions the fragments survived for some weeks and axones (nerve fibres) grew out from the cells. This helped to settle a current controversy about the origin of these structures and incidentally illuminated the potentialities of experimental methods using surviving tissues *in vitro*.

The 'traditional' techniques of tissue culture were rapidly established thereafter. Burrows, studying with Harrison, introduced the use of a plasma clot in place of a lymph clot. Shortly afterwards he became associated with Carrel and they made the joint discovery that embryo extract had a strong growth-promoting effect on certain cells. The technique of growing tissues in plasma clots supplemented with embryo extract then became standard practice. The culture was usually prepared on a coverslip inverted over the cavity of a depression slide, in the manner originally used by Harrison, and the method, elegant in its simplicity, is still in use.

The greatest difficulty in performing tissue culture at that time was the avoidance of bacterial contamination. Alexis Carrel, already a Nobel prize-winner for his work in experimental surgery, was largely responsible for the development of the method in the next few years. Bringing with him a knowledge of aseptic techniques, he tackled tissue culture as he would a surgical operation. In consequence, he was able to obtain consistently successful results and the measure of his technical genius was his feat of keeping a strain of cells in active multiplication for thirty-four years by means of the very tedious methods he had available and in the total absence of antibiotics. Carrel's work demonstrated without doubt that animal cells could be grown indefinitely *in vitro*. Unfortunately, the meticulous surgical techniques he employed dissuaded many biologists from using the method and engendered the belief that tissue culture was fantastically difficult—a belief which is only now being dispelled.

One of the main achievements of the Carrel school was the continuous cultivation of rapidly growing and dividing cells over long periods of time. Success in this field suggested the possibility that cells might be grown almost like protozoa or micro-organisms. Such a thought was a particularly intriguing one and the possible

use of large amounts of cells in metabolic studies occurred to several investigators. The perfection of our present methods of cell culture owes a very great deal to the group at the National Cancer Institute in the United States, headed by Dr. Wilton Earle. This group was the first to grow cells direct on glass in large numbers, the first to grow cultures from single cells and the first to propagate cells intentionally in suspension.

An entirely different approach to the cultivation of tissues *in vitro* was initiated by Dr. Honor Fell. Instead of trying to make cells grow as rapidly as possible, her aim was to maintain small fragments of tissue in a state as close as possible to their state *in vivo*. The 'organ culture' technique, as it is called, is in many ways the most direct descendant of Harrison's original experiment. In the hands of Dr. Fell herself and an outstanding group of workers, especially Dr. Gaillard and Dr. and Mme. Wolff, this technique has yielded a great deal of important information in embryogenesis and endocrinology.

At an early stage in the development of animal tissue culture Warren and Margaret Lewis started to investigate the factors in the medium necessary for growth and survival (1911-1912). Baker, in association with Carrel, also continued to be interested in the composition of the medium and attempted, by analytical procedures, to identify the important constituents present in it. This type of work was carried on by Fischer and then in recent years by a large body of capable workers, especially Parker, Healy, Morgan, White, Waymouth and Eagle, and has resulted in the development of our present-day media.

Plant tissue culture has tended to develop quite separately from animal tissue culture and only in recent years has there been any general exchange of information between the two fields. The idea of the cultivation of plant cells was proposed by Haberlandt but his attempts proved fruitless and in 1902 he abandoned it. First successful attempts were made in 1921 by Molliard and in 1922 by Kotte and Robbins. These workers succeeded in maintaining plant roots for some weeks. Interest in the subject lapsed for a number of years and it was not until White and Gautheret developed suitable media in the mid-1930s that the technique began to appear promising. Subsequently, it advanced very rapidly and synthetic media for the cultivation of plant tissues were developed almost immediately.

The implications of the tissue culture method were not lost, even in the very earliest days, and its potential value in such subjects as morphogenesis, cancer research and virology was immediately recognised, the only discouragement being the difficulties of the technique itself. In spite of these, much valuable information was accumulated by embryologists and histologists from the beginning while experiments in cancer research almost preceded the development of reliable methods. In virology and biochemistry, however, although attempts were made to wrest information from tissue culture material, the technical difficulties proved overwhelming and it is only with recent technical development that its application in these fields has become widespread.

Steinhardt, Israeli and Lambert showed as early as 1913 that the vaccinia virus could survive for several weeks in explanted cornea. No attempt was made to follow up this observation until, in 1925, Parker and Nye demonstrated multiplication of vaccinia virus in tissue cultures of rabbit testis. Similar experiments with vaccinia and also with the Rous sarcoma virus were reported by Carrel and Rivers and Carrel in the next two years.

In 1928 Maitland and Maitland developed a very simple tissue culture method for virus multiplication. This consisted of suspended fragments of tissue in a fluid medium and it led to many interesting studies in the ensuing years. However, it was the observations of Enders and his colleagues in 1948 that gave the subject its great impetus. They showed conclusively that the poliomyelitis virus could be cultivated *in vitro* in the absence of nerve tissue. This observation was made at a time when cell culture techniques had undergone some remarkable developments. With the added practical interest, the number of people in the field increased rapidly and the whole subject has evolved with extraordinary speed in the past ten years. There is now practically no field of biological research in which it has not been successfully employed.

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CURRENT TECHNIQUES

A wide variety of techniques has become available and the main ones in common use are illustrated in Figure 1. One of the simplest consists of embedding small fragments of tissue in a clot on a coverslip inverted over the concavity of a depression slide and sealed to it with paraffin wax. This technique is very easy and is convenient for short term experiments involving large numbers of cultures of small pieces of tissue. By the use of a growth-stimulating medium and frequent transfer, these cultures may be propagated for many generations and it was by this method that Carrel kept his 'immortal' strain alive for almost forty years. However, the maintenance of cells in this manner for long periods of time requires a great deal of practice and patience and it is very difficult to keep cultures growing in a slow-growing state, since this requires that the medium be renewed at frequent intervals without disturbing the tissue. The double coverslip method was developed for use where it was desired to keep tissue alive for long periods without transfer. Techniques using slides with a concavity have the disadvantage that it is impossible to use them with some optical systems and therefore numerous modifications, *e.g.* special phase slides and perforated glass or metal slides, have been developed to permit examination by phase contrast or interferometric microscopy.

Special techniques have been developed for organ culture and in particular Strangeways and Fell developed the watch-glass technique in which the tissue is grown on a clot in a watch-glass which is itself surrounded by wet cotton wool in a Petri dish to maintain humidity. Other organ culture techniques have stemmed from this, in particular the use of a raft of lens paper or rayon net on which the tissue is grown.

Ordinary test-tubes are convenient for growing large numbers of cultures when good optical conditions are not essential. Hence they are used for the maintenance of tissues and for virus and biochemical assays. The tubes may be rotated or kept stationary in racks.

If it is desired to grow large numbers of cultures for subsequent morphological examination, the 'flying coverslip' technique is particularly convenient. Cultures are prepared on narrow coverslips and inserted into roller tubes. It is commonly used in preparing

coverslip cultures for subsequent photography or cinemicrographic study.

A large variety of roller tubes of various kinds has been developed for special purposes. For instance, roller tubes have been made with very thin side windows to permit direct application

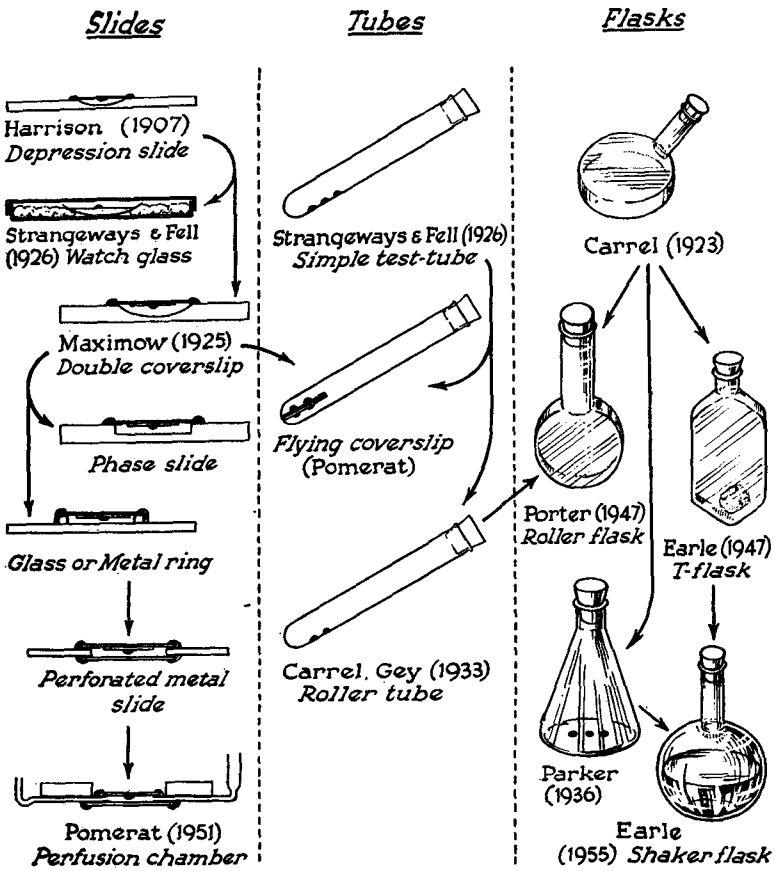

FIG. 1
Vessels used for culturing cells and tissues.

of an end-window Geiger counter. Others have been developed with a micro test-tube at one end so that a chemical analysis of the tissue can be carried out in the same tube after the growth phase is completed.

The original Carrel flask was designed to facilitate the handling of cultures which it was desired to maintain for some time. Very good optical properties were usually insisted upon but nowadays the Carrel flask is used almost entirely to start strains and in this case such good optical qualities are not so necessary. The Carrel flask has otherwise fallen out of use entirely. However, other flasks are used extensively, mainly for growing large quantities of cells. Earle's T flask was designed specifically for this purpose and is specially made to have a very flat surface inside and to have good optical properties. Ordinary Erlenmeyer flasks are also used frequently for growing cells, as are Roux bottles and penicillin culture flasks.

Recently cells have been grown in suspension and originally ordinary round-bottomed flasks, agitated in a special shaker, were used for this purpose. Now there are a number of techniques, some employing stirrers and some employing fast roller tubes.

Besides the techniques described, many others have been employed. Some special vessels have been developed and in particular a number of different perfusion chambers have been described which permit the continuous observation of cells by time-lapse cinemicrography while the medium is being changed.

All of these techniques have been widely applied in biological research. While many of them have been employed especially in particular fields there is no single technique which can be described as useful solely in tackling one kind of problem and most of them have proved valuable in several entirely different sets of circumstances. For instance, Ross Harrison's original method of growing fragments of tissue in a plasma clot proved useful many years later in making the first electron microscopic studies on animal cells. Similarly, organ culture technique, a classical tool of the experimental embryologist, has recently been used to investigate host-parasite relationships in virus studies.

It is this fact, along with the common principles involved in all tissue culture work, that serves to unite the many different techniques as a single branch of biological technology.

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PART I
PRINCIPLES OF CELL CULTURE

CHAPTER II

THE CELL

THIS outline of our present knowledge of the structure and function of cells and tissues is essentially elementary. It is intended mainly for the many non-cytologists who are now using tissue cultures.

The cell is the unit of structure of all metazoa. Metazoa are organisms consisting of large aggregates of cells of different types which live together to the advantage of them all. This implies a certain degree of mutual dependency which is not very highly developed in the lowest metazoa but is very highly developed in the highest forms. Thus it is commonplace that the destruction of any one of the vital organs in an animal will result in the death of the whole. With increasing complexity of organisation masses of different cell types tend to become localised and to form recognisable patterns. An aggregation of cells forming a definite pattern in this way is referred to as a tissue. At a still higher level of organisation tissues aggregate in a characteristic way to form organs. The whole animal consists of an orderly arrangement of organs.

With the exception of a very few creatures at the borderline between protozoa and metazoa, it is unlikely that metazoan cells ever survive for any length of time outside the animal except in tissue culture conditions.

Fine cell structure

The cell is itself a highly organised structure and in many respects it resembles very closely certain protozoa, especially the amoebae. It is surrounded by a membrane, very thin in some cells, thick in others. Plant cells are distinguished from animal cells by having a cellulose cell-wall, which renders the structure rigid. Within it there are a number of well-defined structures. These are the nucleus, mitochondria, lysosomes, Golgi apparatus, centrioles,

and, in some cases, inclusions. The remainder of the cell is the cytoplasm.

The nucleus is separated from the cytoplasm by a well-defined nuclear membrane, which appears to be double. It can rotate freely within the living cell and it has been suggested that this movement may be due to the secretion of material into the cytoplasm. Within the nucleus there occur one or more nucleoli. These are rich in

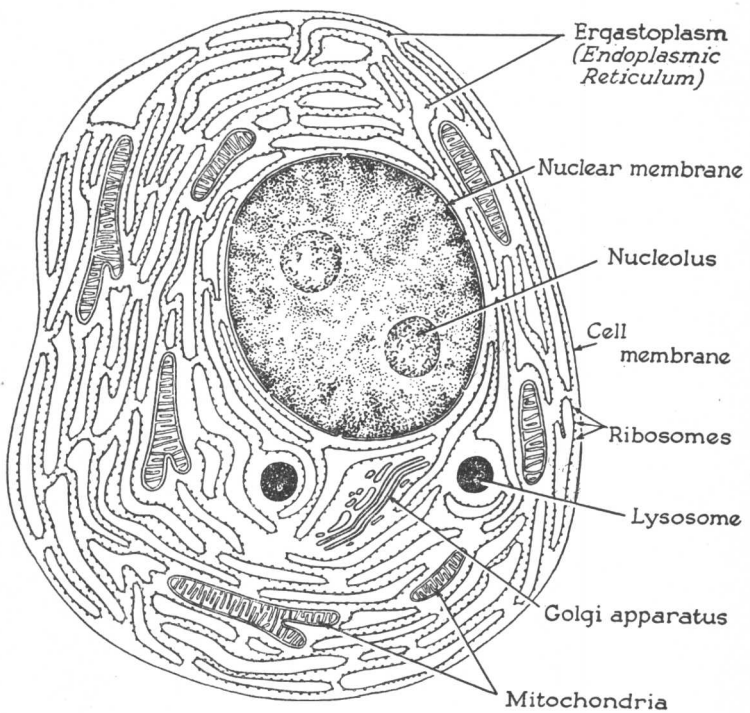

FIG. 2
Diagram of a cell showing its structure.

ribonucleic acid (RNA) and it has been shown that this RNA is metabolically highly active. The remainder of the nucleus normally consists of a structureless basophilic mass, containing a high concentration of deoxyribonucleic acid (DNA) and a characteristic basic protein, nuclear histone. When cell division occurs the structureless material of the nucleus aggregates into a skein of